Plant Physiol. (1997) 115: 375–385

The Differential Expression of Sucrose Synthase in Relation to Diverse Patterns of Carbon Partitioning in Developing Cotton Seed[1]

Yong-Ling Ruan[2], Prem S. Chourey*, Deborah P. Delmer, and Luis Perez-Grau

Program in Plant Molecular and Cellular Biology and Department of Plant Pathology, University of Florida, Gainesville, Florida 32611 (Y.-L.R., P.S.C.); United States Department of Agriculture, Agricultural Research Service, Gainesville, Florida 32611-0680 (P.S.C.); Section of Plant Biology, University of California, Davis, California 95616 (D.P.D.); and Calgene, Inc., 1520 Fifth Street, Davis, California 95616 (L.P.-G.).

Developing cotton (*Gossypium hirsutum* L.) seed exhibits complex patterns of carbon allocation in which incoming sucrose (Suc) is partitioned to three major sinks: the fibers, seed coat, and cotyledons, which synthesize cellulose, starch, and storage proteins or oils, respectively. In this study we investigated the role of Suc synthase (SuSy) in the mobilization of Suc into such sinks. Assessments of SuSy gene expression at various levels led to the surprising conclusion that, in contrast to that found for other plants, SuSy does not appear to play a role in starch synthesis in the cotton seed. However, our demonstration of functional symplastic connections between the phloem-unloading area and the fiber cells, as well as the SuSy expression pattern in fibers, indicates a major role of SuSy in partitioning carbon to fiber cellulose synthesis. SuSy expression is also high in transfer cells of the seed coat facing the cotyledons. Such high levels of SuSy could contribute to the synthesis of the thickened cell walls and to the energy generation for Suc efflux to the seed apoplast. The expression of SuSy in cotyledons also suggests a role in protein and lipid synthesis. In summary, the developing cotton seed provides an excellent example of the diverse roles played by SuSy in carbon metabolism.

The striking feature of SuSy (EC 2.4.1.13) is its potential ability to mobilize Suc into diverse pathways that are important for structural, storage, and metabolic functions of plant cells (Chen and Chourey, 1989; Heinlein and Starlinger, 1989). This enzyme catalyzes a reversible reaction but is thought to preferentially convert Suc into Fru and UDP-Glc (Chourey and Nelson, 1979; Geigenberger and Stitt, 1993; Heim et al., 1993). Hence, SuSy potentially can play an important role in controlling either starch or cellulose biosynthesis by supplying UDP-Glc as a precursor and an immediate substrate, respectively (Chourey et al., 1991; Chourey and Miller, 1995; Delmer and Amor, 1995).

In this context, early studies of maize showed that the deficiency of SS1, one of the two SuSy isozymes, results in a shrunken phenotype of seed with a slightly reduced starch level in the endosperm (Chourey and Nelson, 1976). More recently, transgenic potato plants with antisense inhibition of SuSy gene expression showed a dramatic loss of the enzyme activity and a reduction of tuber starch content (Zrenner et al., 1995). These results show clearly that SuSy affects starch biosynthesis in maize seeds and potato tubers. However, the enzyme is not absolutely essential, since up to 60 to 70% of wild-type levels of starch are still present in the SS1-deficient maize kernel (Chourey and Nelson, 1976), and up to 30 to 80% are present in transgenic potato tubers (Zrenner et al., 1995). In addition to the reduced starch levels in the maize shrunken endosperm, there is also an early cell degeneration characterized by a brittleness of the endosperm cell wall (Chen and Chourey, 1989; Chourey et al., 1991). Thus, it has been suggested that SuSy may also play a role in biosynthesis of the endosperm cell wall by supplying UDP-Glc for cellulose biosynthesis (Chourey et al., 1991; Chourey and Miller, 1995; Carlson and Chourey, 1996). This notion is further supported by biochemical studies of SuSy in cotton (*Gossypium hirsutum* L.) fiber (Amor et al., 1995). Thus, interesting questions emerge concerning the relative contribution of SuSy to cellulose and starch biosynthesis and whether such a dual function coexists in plants.

In this regard, the developing cotton seed represents a unique system in which the potential roles of SuSy in controlling diverse patterns of carbon partitioning can be simultaneously assessed. The cotton seed consists of three tissue types (Fig. 1): (a) the cellulosic fibers derived from the outermost single cell layer of the seed coat epidermis; (b) the seed coat, where phloem terminates; and (c) the embryo, which consists predominately of cotyledons symplastically isolated from the seed coat (Hendrix, 1990). The

[1] This work was supported in part by U.S. Department of Agriculture-Agricultural Research Service and U.S.-Israel Binational Agricultural Research and Development Fund. It was a cooperative investigation between the U.S. Department of Agriculture-Agricultural Research Service and the Institute of Food and Agricultural Sciences, University of Florida. This paper is Agricultural Experimental Journal Series No. R-05717.

[2] Present address: Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, G.P.O. Box 1600, Canberra, ACT 2601, Australia.

* Corresponding author; e-mail psch@gnv.ifas.ufl.edu; fax 1–352–392–6532.

Abbreviations: CF, 5(6)-carboxyfluorescein; CFDA, 5(6)-carboxyfluorescein diacetate; DPA, days postanthesis; SuSy, Suc synthase.

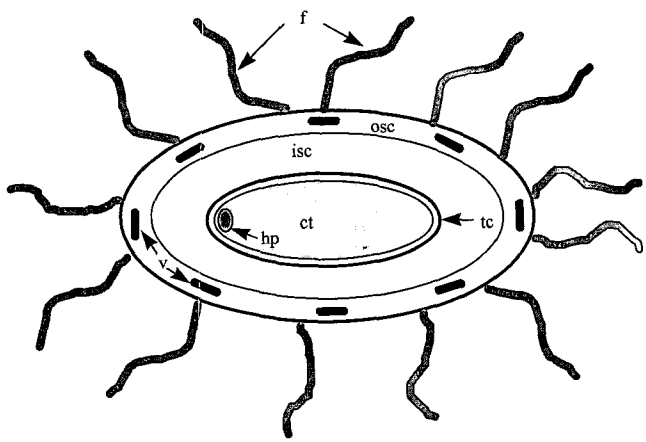

Figure 1. A schematic representation of a developing cotton seed. ct, Cotyledon; f, fiber; hp, hypocotyl; isc, inner seed coat; osc, outer seed coat; tc, transfer cell; v, vascular bundle.

fiber secondary cell wall formation starts from about 15 DPA and is characterized by massive deposition of cellulose, which constitutes more than 90% of the dry weight of the mature fiber cell (Basra and Malik, 1984; Delmer and Amor, 1995). The seed coat, on the other hand, accumulates starch, whereas the cotyledons deposit protein and oil coincidentally with the rapid phase of fiber cellulose biosynthesis (Doman et al., 1982; Trelease, 1986; Hendrix, 1990). Thus, multiple metabolic pathways for carbon use are evident in the developing cotton seed. Based on enzyme activity assay, SuSy appears to be the major enzyme, as compared with invertase, in degrading unloaded Suc in cotton seed (Hendrix, 1990). However, little is known about its spatial expression in this complex system except for the observation of its localization in the fibers (Amor et al., 1995; Nolte et al., 1995). It is important to understand how SuSy expression at the cell level could potentially control the multidirectional post-phloem Suc transport in these seeds. To this end, in vitro feeding of detached fibers with [^{14}C]Suc results in rapid incorporation of ^{14}C label into newly synthesized cellulose (Amor et al., 1995). This indicates the potential role of SuSy in controlling cellulose biosynthesis of cotton fiber (Amor et al., 1995; Delmer and Amor, 1995). However, whether SuSy expression in vivo correlates with the developmental profile of fiber cellulose biosynthesis remains to be demonstrated. Similarly, a potential role for SuSy in starch biosynthesis in developing cotton seed is also unknown.

Although cotton is the most important textile fiber crop, molecular studies of this plant are scarce compared with other major crops (Ma et al., 1995). Here we present the results of a study of SuSy gene expression in cotton seeds at both the molecular and cellular level in relation to its role in controlling carbon flow to diverse biosynthetic processes in vivo. Attention was focused on the cotton seed during the rapid phase of sink activity; in particular, we focused on fiber cellulose biosynthesis 16 to 18 DPA, unless specified otherwise. The results obtained show that, unlike many other sink tissues, SuSy appears not to be essential for starch production but rather could be important in fiber cellulose biosynthesis. Furthermore, several lines of evidence indicate that this enzyme could play an important role in controlling post-phloem Suc mobilization to fiber and cotyledonary cells for biosynthesis of cellulose and protein/oil, respectively, hence providing significant insight into how Suc metabolism and transport are coordinated.

MATERIALS AND METHODS

Cotton (*Gossypium hirsutum* L.) seeds were sown in a potting mixture (Metro-Mix 300 growing medium, Scotts, Columbus, OH). The plants were raised under greenhouse conditions with partial temperature control (25–30°C during the day and 18–22°C during the night). About 100 g per pot of Osmocote (Scotts), a controlled release fertilizer with N:P:K at 1:1:1, was applied once every 20 d. The plants were watered once every 2 d. Standard pest and disease control practices were used. Cotton fruit age was determined by tagging the flowering truss when the flower was fully opened. All samples, unless otherwise specified, were frozen in liquid N_2 and then stored at −80°C until analysis. The frozen developing cotton seed was further separated into fiber, seed coat, and cotyledon on dry ice before experimentation.

cDNA Library Construction and Screening

The λZapII cDNA library was prepared from cDNA derived from poly(A^+) mRNA isolated from fibers of cotton bolls at approximately 21 DPA. A pair of degenerate oligonucleotides, primer A (5′-TT[G/T]GAGA[A/G]-GGG[A/T/G]TGGGG) and primer B (5′-AAGC[G/T][T/A/G]GAGATCCATTTGCG), based on regions of homology between described SuSy protein sequences, was used in PCR to generate a 450-nucleotide fragment of the cotton fiber SuSy sequence. The template for the reaction consisted of phage DNA extracted from an aliquot of the cotton fiber cDNA library.

The PCR product was used as a probe to screen the above-mentioned cDNA library at moderate stringency. The blots were hybridized in 6× SSC, 0.02 M NaCl, 1.0% SDS, 5× Denhardt's solution, and 10 mg mL^{-1} salmon sperm DNA at 56°C. Posthybridization washes were performed three times for 30 min each in 5× SSC and 0.5% SDS at 56°C and then with the same wash duration in 0.5× SSC and 0.5% SDS at 50°C. The five strongest positive hybridization phage plaques were isolated and purified according to standard procedures. The phagemid containing the cDNA was excised from these phages according to the instructions provided by Stratagene in the λZapII manual.

DNA and RNA Gel Blots

Genomic DNA was isolated from 7-d-old dark-grown seedlings essentially as described by Dellaporta et al. (1983). To remove phenolic compounds from the sample, 10% (w/v) of soluble PVP (M_r 40,000) was added to the extraction medium and 2% was added to the subsequent phenol/chloroform purification. Approximately 10 μg

of DNA was digested with one of four restriction enzymes according to the manufacturer's specifications (GIBCO-BRL). The digested samples were fractionated on 0.6% agarose gels, transferred to a Nytran membrane (Schleicher & Schuell), and prehybridized in 50 mM Pipes (pH 6.5), 100 mM NaCl, 50 mM sodium phosphate (pH 6.5), 1 mM EDTA (pH 8.0), and 5% SDS. The blots were hybridized in the same solution with ^{32}P-labeled SS3 cDNA probe (3×10^6 cpm/mL) overnight at 65°C. Blots were washed two times for 45 min each in 6× SSC (0.5 mM EDTA, pH 8.0, 5 mM sodium phosphate, pH 6.5, and 5% SDS) and two times for 30 min each in 0.2× SSC in the same wash solution but with 1% SDS. The blots were exposed to x-ray film for 3 d at −70°C with intensifying screens.

For RNA gel blots, total RNA was isolated as described by Wadsworth et al. (1988). This protocol was modified for removal of extremely high levels of phenolic compounds in green cotton tissues and seeds by adding 0.2 mM Glc (as a reducing agent) and 10% (w/v) soluble PVP (M_r 40,000) to the extraction medium. To increase yield, incubation with 6 M LiCl was extended to 2 h on ice. The RNA was glyoxylated and fractioned on a 1.2% agarose gel and transferred to a Nytran membrane. The blots were hybridized and washed as described above for DNA gel blots.

Enzyme Activity Assay

Samples (approximately 0.5 g each) were ground to a fine powder in liquid N_2. The grinding continued for 5 min in cold extraction buffer (3:1, v/w) containing 25 mM Hepes-KOH (pH 7.3), 5 mM EDTA, 1 mM DTT, 0.1% soluble PVP (M_r 40,000), 1 mM PMSF, and 0.01 mM leupetin. The homogenate was centrifuged at 10,000*g* for 5 min at 4°C. The supernatant was dialyzed against the extraction buffer overnight at 4°C. The protein concentrations were determined using the Bio-Rad *DC* protein assay kit with BSA as a standard. SuSy activity was measured as the rate of Suc cleavage (Chourey, 1981). The resultant reducing sugars were estimated according to the method of Nelson (1944).

Protein Gel Blots

Total soluble protein was isolated as described above for the enzyme activity assay, denatured by SDS and boiling treatments. The denatured protein samples were separated on SDS-PAGE minigels (10%) according to the method of Laemmli (1970). Electrophoresis and blotting were performed as described in the instruction manuals for the Bio-Rad Mini-Protein II electrophoresis cell and trans-blot electrophoretic transfer cell. Cotton SuSy antibody preparation has been detailed by Amor et al. (1995). Briefly, membrane-associated SuSy protein p91 was isolated from fibers 21 DPA. A total of 200 μg of purified protein was injected into rabbits four times (50 μg each time). The serum from the third or fourth venapuncture was collected, spun at 100,000*g*, and used at a 1:1500 dilution. SuSy antigen blotted on the nitrocellulose membrane (Schleicher & Schuell) was detected via chemiluminescence using the *SuperSignal CL-HRP* substrate system detection kit (Pierce) according to the manufacturer's instructions. Briefly, the membrane was blocked in 5% nonfat dry milk in PBST (PBS-Tween 20) for 1 h. After the membrane was washed with PBST, it was incubated with polyclonal antibody against cotton SuSy (1:1500 dilution) for 1 h. After the first wash for 15 min, followed by four 5-min washes, the membrane was incubated with anti-rabbit, horseradish peroxidase-conjugated, secondary antibody (1:5000 dilution) for 1 h. After four washes, the membrane was treated with chemiluminescence reagent for 1 min prior to exposure to x-ray film.

Immunolocalization and Histochemical Staining

Immunolocalization was conducted according to the procedure described by Chen and Chourey (1989) and the modifications described by Cheng et al. (1996). To avoid physical contamination of the seed sections by fiber due to its softness, the fiber was removed from the remaining seed and treated in parallel. Briefly, tissues were sectioned (12 μm), affixed to slides, deparaffinized, rehydrated, and washed with PBS. Slides were then incubated with 1:1500 diluted SuSy polyclonal antibody or preimmune serum in a humid environment overnight. After the slides were washed with PBS, they were incubated for 20 min in a solution of secondary antibody consisting of biotinylated anti-mouse anti-rabbit immunoglobulin and alkaline phosphatatase-labeled streptavidin (LSAB2 kit, Dako, Carpinteria, CA). Signal was visualized using NEW Fuchsin Chromogen (Dako), which resulted in a precipitate of fuchsia-colored end product at the site of the antigen. Pairs of immuno- and preimmunostained sections were treated on the same slide for better comparison.

Sections adjacent to those used for the immunolocalization were subsequently stained with Fast Green/IKI (Sigma) to localize starch granules. After deparaffinization in xylene and ethanol, the sections were stained with Fast Green for 2 min. The sections were placed for 6 s in a rinse solution containing 25% clove oil, 33% xylene, and 40% ethanol, followed by a 10-s wash with 90% xylene plus 10% ethanol. The sections were incubated with IKI solution for 2 min and then washed with water to remove excess IKI. The sections were air-dried for 1 h, placed in xylene for 10 min, and mounted with Permount (Fisher-Scientific).

Transport Studies with CFDA

The application and visualization of symplastic fluorescent dye CF were performed essentially as described by Ruan and Patrick (1995). In brief, the membrane-permeant, nonfluorescent dye CFDA (Sigma) was prepared as a 2.0% (w/v) stock solution in acetone and stored at −20°C. Before use it was diluted to 0.005% (w/v) with a 5 mM Mes-Tris (pH 6.0) buffer containing 20 mM KCl, 0.5 mM $CaCl_2$, 0.2% (w/v) BSA, and 0.2% (w/v) PVP. The osmolality of the solution was adjusted to 150 mosmol/kg with sorbitol. Direct loading of seed coats with CFDA was carried out using freshly sampled cotton seed coat halves 16 DPA. The seed samples were prepared by removing seed from harvested bolls, detaching fibers from seeds, and cutting transversely around the integumentary fusion line. The result-

ing coat halves were removed gently from the enclosed embryo. The dye solution was introduced into the seed coat cavity vacated by the cotyledons. Dye flow driven by evaporative loss from the seed coat was minimized by placing the samples in a water-saturated atmosphere. After specified loading times, dye was removed from the coat apoplast by three 3-min washes with 15 mL of the buffer solution at 4°C. Thereafter, the dye-loaded coats were transferred to 20 mL of buffer solution held at 25°C in a shaking (45 oscillations min^{-1}) water bath. This served to capture any dye molecules that escaped to the coat apoplast. Hence, under these conditions, any dye movement can be attributed to passage through the coat symplast. Following a 1- or 2-h incubation period, free-hand sections were cut from the coats and mounted in the buffer solution. The fluorescence of CF in the coat symplast was observed as described previously (Ruan and Patrick, 1995).

Sugar and Starch Analysis

Fresh samples, about 0.2 g each, were weighed and ground to a fine powder in liquid N_2. Soluble sugars were extracted by boiling each sample in 10 mL of distilled water for 3 min and then by incubation in a 80°C shaking water bath for 2 h. Suc, Glc, and Fru were measured enzymatically after extraction from seed tissues (Ruan et al., 1995). The recovery rate of the standard Suc and hexoses were about 94% with this procedure. Starch was determined using a starch assay kit (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, starch was extracted using DMSO and hydrolyzed to Glc by amyloglucosidase. The residual Glc in the tissues was estimated from samples without amyloglucosidase treatment. Starch levels were measured as Glc equivalents and expressed according to the values derived from a set of starch standards.

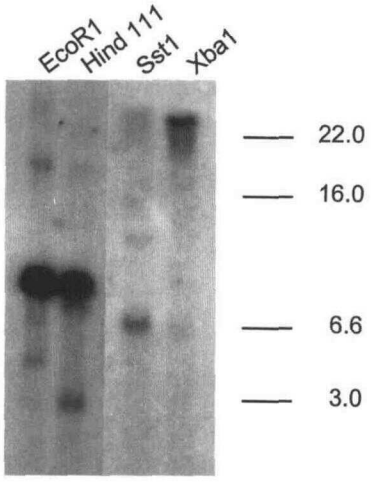

Figure 2. Genomic DNA gel-blot analysis with SS3. Each lane contains 10 μg of genomic DNA digested with one of four indicated restriction enzymes. Numbers at right indicate lengths of DNA in kilobase pairs.

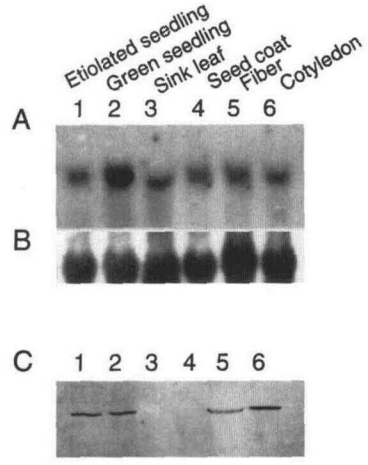

Figure 3. Differential expression of the SuSy gene at the RNA and protein levels. A, RNA gel blot with 15 μg of total RNA in each lane. B, The same blot sequentially hybridized with a maize rRNA probe. C, Protein immunoblot with 50 μg of protein in each lane from crude extracts of the same samples as used for the RNA gel-blot analysis. The numbers correspond to the lane in A.

RESULTS

cDNA Cloning and Genomic DNA Gel Analysis of SuSy

To initiate a SuSy expression study in cotton, several partial-length SuSy cDNA clones were isolated from a cotton fiber cDNA library using a 450-bp PCR-generated fragment as a probe. The longest clone, designated SS3 (GenBank accession no. U73588), was 2.2 kb. Sequence analysis showed that SS3 shares 76 to 80% of their nucleotide identities with the gene from radish, faba bean, tomato, and potato. Figure 2 represents a genomic DNA gel blot with SS3, showing multiple fragments generated by each of four restriction enzymes. This suggests that a small SuSy gene family might exist in cotton.

Tissue- and Cell-Specific Expression of SuSy and Its Relation to Starch and Cellulose Biosynthesis

Several representative sink tissues were selected for RNA gel-blot analysis. Figure 3A shows that SuSy RNA was detectable in all of the tissues. The steady-state level of SuSy mRNA was more abundant in green seedlings than in etiolated seedlings. The transcript size appeared to be slightly reduced in developing leaves, suggesting that a different SuSy gene might exist in this tissue. Finally, in the developing seed there was nearly equal abundance of SuSy mRNA in the fiber, seed coat, and cotyledons. Figure 3B represents rRNA as a loading and transfer control of the total RNA.

SuSy expression in these same tissues was also examined at the protein level by SDS-immunoblot analyses using polyclonal antibodies raised against cotton SuSy. To test the detection limit of the SuSy protein by the antibody, a series of diluted crude extracts from fibers were used. An SuSy protein was readily detectable from 1.0 μg of total soluble protein loaded (data not shown). Unlike

the pattern seen for mRNA level in Figure 3A, the steady-state level of SuSy protein in green seedlings was similar to that of etiolated seedlings (Fig. 3C). A surprising observation was that the SuSy protein was undetectable in the seed coat and young leaves, even though these tissues have shown detectable levels of SuSy mRNA (Fig. 3, C versus A). However, SuSy protein was clearly detectable in fibers and cotyledons (Fig. 3C). We also examined SuSy activities from the three different parts of developing cotton seed. The specific activity of the soluble enzyme was 147 ± 12 and 122 ± 10 nmol reducing sugar mg^{-1} protein min^{-1} in fibers and cotyledons, respectively, but was undetectable or in trace levels (<5% of that in fiber) in the seed coat.

To gain an insight into the cellular location of the enzyme and its potential role in cotton seed development, SuSy protein was immunolocalized on tissue sections of cotton seed 16 to 18 DPA (Fig. 4). A positive signal for the SuSy protein, as evidenced by an intense fuchsia-colored reaction product, was readily detectable in fiber and cotyledon (Fig. 4, B and E, respectively). The most intense signal was seen in transfer cells at the innermost layer of the seed coat (Fig. 4E). At high magnification (Fig. 4G), this single-cell layer of transfer cells was not only enriched in SuSy protein but also characterized by having thickened cell walls. No SuSy signal was seen in most of the seed coat cells, including the unloading region around the vascular bundle (Fig. 4E), or in the respective tissues treated with the preimmune serum (Fig. 4, A and D). The same localization pattern was also seen in seed sections 8 to 10 DPA (data not shown). The yellow-brown color seen in the seed coat of sections treated with either preimmune serum (Fig. 4D) or polyclonal antibodies (Fig. 4E) is due to the seed coat pigment.

Serial sections from the same seeds were also stained with IKI to test whether the cell-specific SuSy localization in the developing cotton seed correlated with starch deposition. The results presented in Figure 4, C, F, and H, show that starch was exclusively localized in seed coat cells where SuSy was undetectable or was found in trace levels (Fig. 4, E versus F). On the other hand, starch was not detectable in fibers and the transfer cells of the seed coat or in cotyledons where SuSy was abundant (Fig. 4, C, F, and H versus B, E, and G). Examination of seed sections 8 to 10 DPA revealed a similar localization pattern except that starch staining was weak in the seed coat cells as compared with that 16 to 18 DPA in Figure 4, F and H (data not shown).

To examine whether the expression of SuSy protein in fibers (Figs. 3C and 4B) correlates with the rates of cellulose biosynthesis in these cells, the levels of soluble SuSy protein and activity were determined as a function of fiber development. Figure 5A shows that the onset of the secondary cell wall cellulose synthesis about 16 DPA coincided with the highest level of SuSy protein, as judged from the intensity of the polypeptide in the immunoblot, and the polypeptide became undetectable after the secondary wall synthesis ceased. A similar pattern was seen in the developmental change of soluble SuSy specific activity, which increased about three times from the phase of primary cell wall biosynthesis to the onset of secondary cell wall biosynthesis and remained relatively high until fiber maturation (Fig. 5B).

The Cellular Pathway of Photoassimilate Transport in Cotton Seed Coat: Visualization with a Symplastic Fluorescent Dye

To understand how the cell-specific expression of SuSy could potentially mobilize photoassimilate to fibers and cotyledons, we examined the functional symplastic continuity of the developing cotton seed coat by using a membrane-impermeant fluorescent probe, CF. The principle of this technique is based on the fact that the nonfluorescent dye CFDA, upon entering a cell, is cleaved by cytoplasmic esterases to produce the membrane-impermeant fluorescent molecule CF (Goodall and Johnson, 1982). In this form it can be used to monitor the continuity of the symplast (Patrick and Offler, 1995; Ruan and Patrick, 1995). Figure 6 shows that CF, initially loaded as CFDA into the empty cavity vacated by an embryo, entered the innermost cells of the seed coat (Fig. 6A) and moved extensively through the inner to the outer seed coat after 1 and 2 h, respectively (Fig. 6, B and C). Moreover, the dye readily moved into fibers from the interconnecting seed coat cells as demonstrated in Figure 6D. We also noted that the long- and narrow-shaped cell layer that interconnects the outer and inner seed coat seen in Figure 4F was readily permeable to the dye (Fig. 6, B and C).

Table I shows that the Suc concentration in seed coats was about 10 times higher than in fibers and cotyledons, whereas Glc and Fru levels were highest in fibers. To verify the qualitative IKI-staining data in Figure 4, C, F, and H, starch was also quantified enzymatically for the three tissue types. As shown in Table I, starch was predominantly detected in the seed coat, with only marginal levels in fiber and cotyledon fractions.

DISCUSSION

SuSy May Not Be Essential for Starch Biosynthesis in Developing Cotton Seed

One significant observation in this study, in contrast to reports in other plants, is that the starch-accumulating cells in developing cotton seeds showed no detectable levels of SuSy protein during the rapid phase of cellulose biosynthesis (Figs. 3 and 4; Table I). Although a formal possibility of extremely low levels of SuSy in those starch-accumulating cells cannot be excluded, the following observations make such a possibility unlikely. First, we used a polyclonal antibody raised against cotton SuSy, which should recognize all SuSy isozyme(s) present in the tissue. In addition, SuSy specific activity was, at most, in trace levels in the seed coat tissue. The trace level detected may well be due to SuSy activity from the one layer of transfer cells at the innermost seed coat (Fig. 4). Moreover, SDS-immunoblots failed to show SuSy protein in 50 μg of total protein from seed coat extracts, but the same protein was readily detectable from 1 μg of fiber protein. Thus, the level of SuSy protein was at least 50 times lower in seed coats

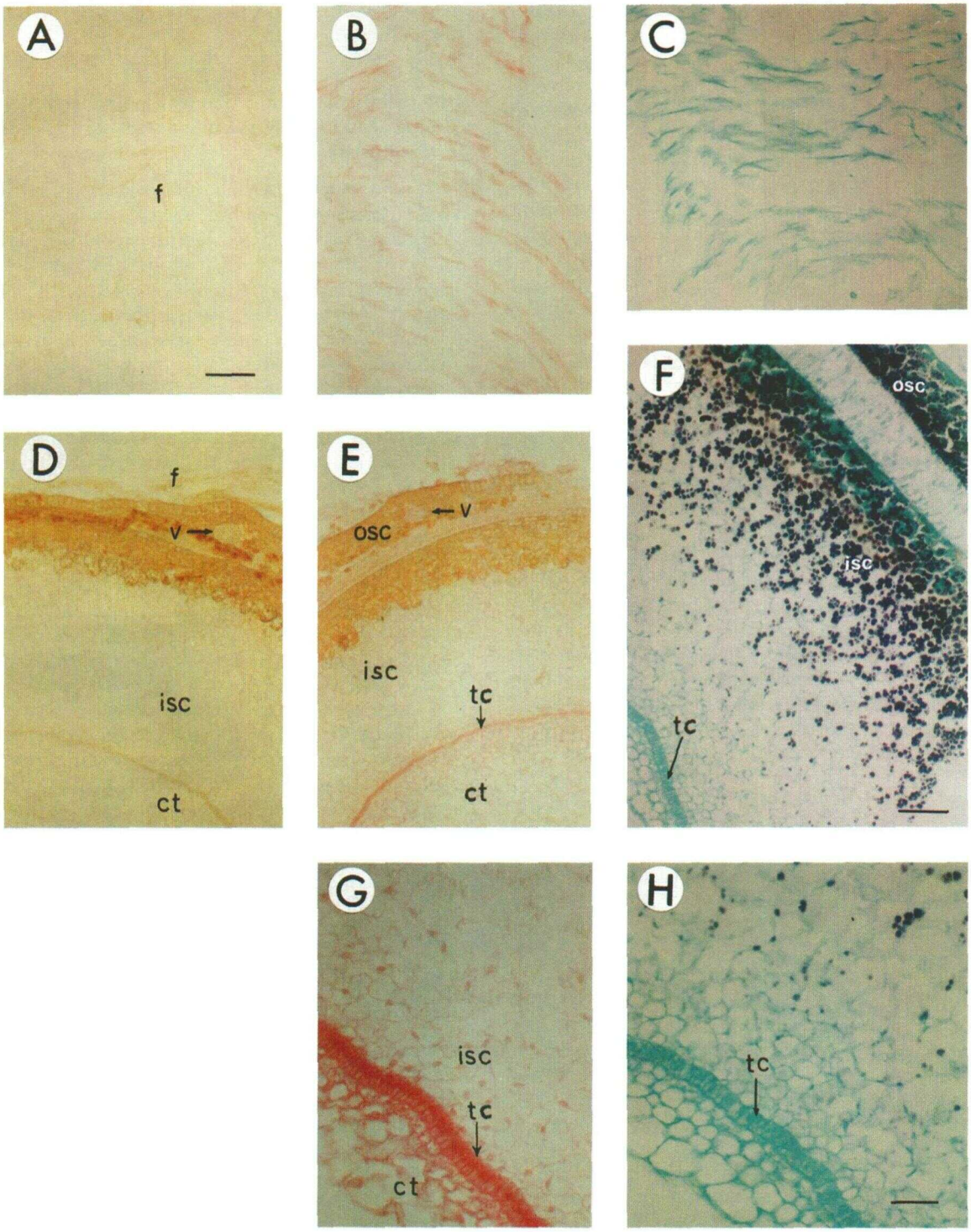

Figure 4. In situ co-localization of SuSy protein and starch in developing cotton seed. The red and black signals represent SuSy protein and starch, respectively. A and D, Treated with preimmune serum. B, E, and G, Treated with polyclonal antibody against cotton SuSy. C, F, and H, Fast Green/IKI staining. G and H, Magnified views of the interface area between the seed coat and cotyledon. Note the strong signal of SuSy protein (G) but deficiency of starch (H) in the transfer cells at the innermost seed coat and in adjoining cotyledon cells. Bars (in μm) = 270 in A; 135 in F; 65 in G and H. The scale in B, C, D, and E is the same as in A. ct, Cotyledon; f, fiber; isc, inner seed coat; osc, outer seed coat; tc, transfer cells; v, vascular bundle.

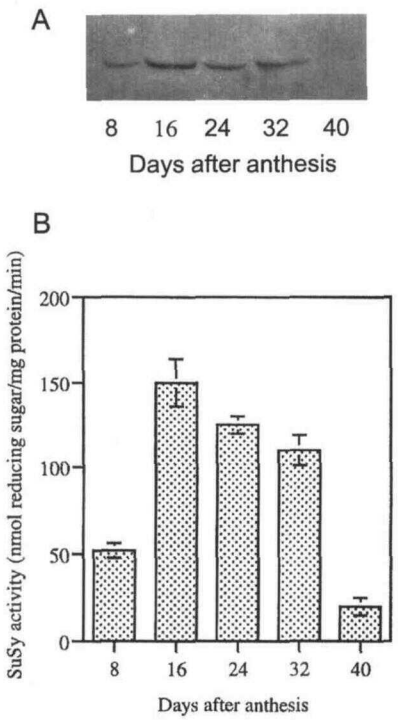

Figure 5. Developmental profile of SuSy protein and activity in developing cotton fibers. A, Protein immunoblot analysis shows the SuSy polypeptide in fiber extracts during different developmental stages. B, SuSy specific activity in developing cotton fiber. Each value represents the mean ± SE of four replicates. Classification of fiber developmental stages: 0 to 15 DPA, Primary cell wall synthesis; 16 to 21 DPA, transition phase; 22 to 32 DPA, secondary cell wall synthesis; >32 DPA, maturation.

than fibers. The starch levels in the seed coat (Table I) were in the same range as in the developing maize kernel (Y.-L. Ruan and P.S. Chourey, unpublished results) and cotyledons of *Vicia faba* (Weber et al., 1996). The high level of starch in the expanding seed coat cells is apparently due to its rapid biosynthesis at the stage examined, since the starch level of this tissue 16 to 18 DPA was doubled when compared with that 8 to 10 DPA (15.6 and 7.0 mg of starch per seed coat, respectively). This, together with the fact that SuSy protein was undetectable in this tissue from 8 to 18 DPA, strongly suggests that the primary path to channel carbon for starch biosynthesis in developing cotton seed coat cells is independent of SuSy.

This conclusion is important for several reasons. First, to the best of our knowledge, a contrasting localization pattern of SuSy and starch accumulation in nonphotosynthetic tissues has not been reported previously. SuSy gene expression has been shown to be positively correlated with starch accumulation at the cell level in developing tomato fruit (Wang et al., 1993, 1994) and maize endosperm (Chen and Chourey, 1989; Heinlein and Starlinger, 1989) and at the tissue level in developing cotyledons of *V. faba* (Heim et al., 1993). These results are consistent with the view that SuSy plays an important role in controlling starch biosynthesis (Chourey and Nelson, 1976; Chen and Chourey, 1989; Wang et al., 1994; Zrenner et al., 1995). Whether the contrasting pattern of SuSy expression and starch deposition observed in this study is unique to cotton seeds or exists in other plant tissues warrants further investigation. It is noteworthy that recent studies have demonstrated that SuSy activity does not correlate with starch accumulation in developing canola seeds (King et al., 1997).

One obvious question relates to the pathway by which starch is synthesized in the absence of SuSy in developing cotton seeds. In this context it is generally believed that the overall reaction from Suc to starch via SuSy involves the PPi-dependent cleavage of UDP-Glc to produce Glc-1-P, which is used for the formation of ADP-Glc, the immediate substrate of the different isoforms of starch synthase (Kleczkowski, 1994; Koßmann et al., 1995). In the absence of SuSy, however, cotton seed coat cells might derive UDP-Glc from SuSy-enriched fiber cells via a symplastic path-

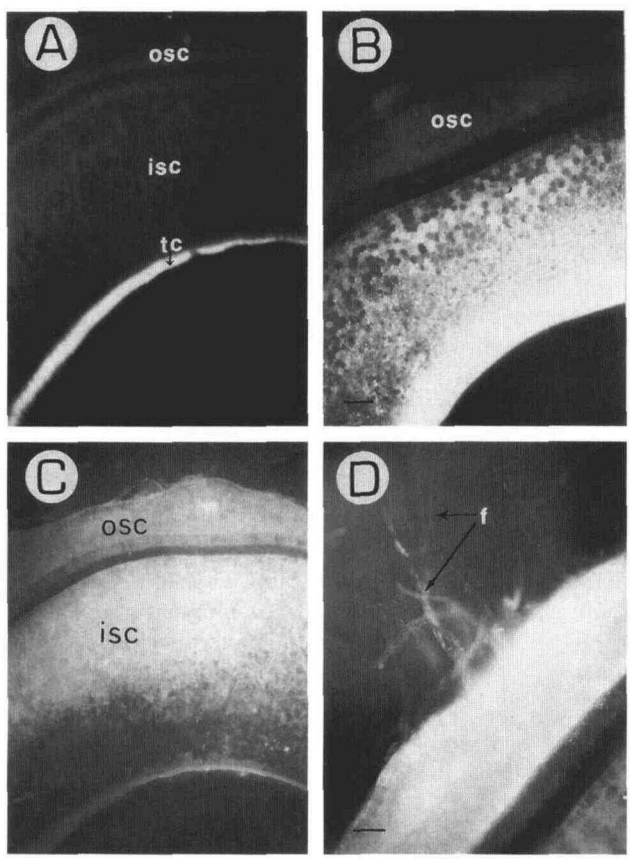

Figure 6. Light-fluorescent micrographs of hand-cut transverse sections of a developing cotton seed coat loaded at the innermost cell layer with the membrane-impermeable fluorescent dye CF. A, The dye was restricted to the transfer cell region at the innermost cell layer of the seed coat 0 h after exposing this side of the seed coat to a nonfluorescent, membrane-permeable dye CFDA for 20 min. Note the weak autofluorescent intensity at the boundary between inner and outer seed coat. Bar = 200 μm. B, The dye moved throughout the inner seed coat after a 1-h incubation in a buffer solution. Bar = 200 μm. C, The dye moved up to the outer seed coat after a 2-h incubation period. Bar = 200 μm. D, The magnified view of the outermost side of the seed coat in C, showing that CF moved into fiber cells after a 2-h incubation in the buffer solution. Bar = 65 μm. f, Fiber; isc, inner seed coat; osc, outer seed coat; tc, transfer cell.

Table I. *Levels of soluble sugars and starch in developing cotton seed*
Values represent means ± SE of four replicates.

Tissue	Suc	Glc	Fru	Starch
		mM		mg g^{-1} fresh wt
Seed coat	49.6 ± 1.4	38.1 ± 3.4	43.4 ± 3.8	39.8 ± 1.0
Fiber	5.8 ± 0.1	78.1 ± 6.2	70.4 ± 6.4	2.8 ± 0.3
Cotyledon	4.8 ± 0.2	50.8 ± 2.0	42.0 ± 1.8	5.1 ± 0.2

way (Figs. 3, 4, and 6). This is unlikely since UDP-Glc in fibers is used predominantly for secondary cell wall cellulose biosynthesis at the stage examined here (Carpita and Delmer, 1981). Alternatively, it is possible that the seed coat cells could generate Glc-1-P for starch biosynthesis through Suc cleavage by vacuolar acid invertase (Hendrix, 1990). Synthesis of Glc-1-P from vacuolar Suc was proposed previously by ap Rees (1988). Consistent with this hypothesis is the rapid turnover of the vacuolar pool of Suc (Borland and Farrar, 1988) and the high flux of carbon between cytosol and vacuole in some plants (Sonnewald et al., 1991). Indeed, expression of yeast-derived invertase in the vacuoles of transgenic tobacco plants results in higher starch accumulation relative to the control plants (Sonnewald et al., 1991).

The molecular basis for the deficiency of SuSy protein in the cotton seed coat (Fig. 3C) appears to be posttranscriptional down-regulation of the gene. This is evidenced by the near-equal abundance of SuSy mRNA in this tissue as compared with fiber and cotyledons (Fig. 3, A and B). A similar posttranscriptional control in SuSy gene expression is also seen in cotton sink leaves and light-grown seedlings that have increased levels of SuSy mRNA compared with dark-grown seedlings but similar levels of protein (Fig. 3, A and C). Posttranscriptional control appears to be a common model for regulation of SuSy gene expression. It has been described previously in anaerobically treated maize seedlings (McElfresh and Chourey, 1988; Taliercio and Chourey, 1989), during normal development in maize embryos (Chourey and Taliercio, 1994), in developing cotyledons of *V. faba* (Heim et al., 1993), and in tomato fruit (Wang et al., 1994).

SuSy Is Essential for Cellulose Biosynthesis in Developing Cotton Fibers

In contrast to the lack of a relationship between SuSy and starch accumulation, the results described here strongly suggest that SuSy plays a critical role in cellulose biosynthesis of cotton fibers by supplying UDP-Glc as a substrate (Amor et al., 1995; Delmer and Amor, 1995). We have shown that SuSy was not only immunolocalized in fiber cells (Figs. 3 and 4), confirming previous observations by Amor et al. (1995) and Nolte et al. (1995), but also enzymatically active. More importantly, the developmental changes of the SuSy protein level and specific activity in fibers were highly correlated with the profile of secondary wall cellulose biosynthesis (Fig. 5). The relatively high levels of hexose in the fiber (Table I) are also in agreement with the role of SuSy in degrading Suc for cell wall synthesis. Additional evidence for SuSy-controlled cellulose biosynthesis comes from the feedback inhibition of SuSy (*Sh*) promoter expression by suppression of cellulose biosynthesis in a transient expression system in maize (Mass et al., 1990).

It is noted that in developing cotton fiber about one-half of the total SuSy is tightly associated with the plasma membrane (Amor et al., 1995). Following this finding, Delmer and Amor (1995) proposed a model in which some form of SuSy might be associated with the cellulose synthase complex and serve to channel carbon directly from Suc via UDP-Glc to the complex. However, the role of soluble SuSy in fiber development is unknown. The observation that the soluble SuSy is positively associated with the developmental profile of fiber cellulose biosynthesis (Fig. 5) indicates that the soluble and membrane-associated form of SuSy may be functionally coupled. Physiologically, a tight coupling between the two could be vital for the overall development of cotton fiber based on the following considerations. First, the soluble form may compensate with the plasma membrane-associated SuSy in generating sufficient UDP-Glc, which is in high demand during the secondary cell wall biosynthesis (Carpita and Delmer, 1981; Basra and Malik, 1984; Amor et al., 1995). Second, cytoplasmic SuSy could play a role in generating energy for synthesis of RNA, protein, and lipids during active fiber growth (Basra and Malik, 1984).

Coordination between Suc Metabolism and Transport Mediated by SuSy

The massive cellulose biosynthesis in cotton fibers requires an efficient, continuous supply of photoassimilate (Ryser, 1992). Structural studies have shown that at the stage of secondary cell wall biosynthesis, the base of the fiber cell wall is suberized, thus preventing photoassimilate transport to the fiber through the apoplast (Ryser, 1992). On the other hand, the high frequency of plasmodesmata connecting the seed coat and fiber cells could account for the import of assimilate to fibers via a symplastic pathway (Ryser, 1992). However, the functionality of the plasmodesmata has not been demonstrated (Buchala, 1987; Ryser, 1992). We show here that CF moved readily from the innermost layer of the seed coat through many layers of inner and outer seed coat cells, and finally into the fibers (Fig. 6). This indicates that a functional symplastic pathway exists across the entire seed coat tissue. Significantly, movement kinetics of CF in sink tissues have been demonstrated to be similar to that of ^{14}C-labeled soluble sugars (Ruan and Patrick, 1995). Therefore, our observation, together with the cytological studies of Ryser (1992), strongly

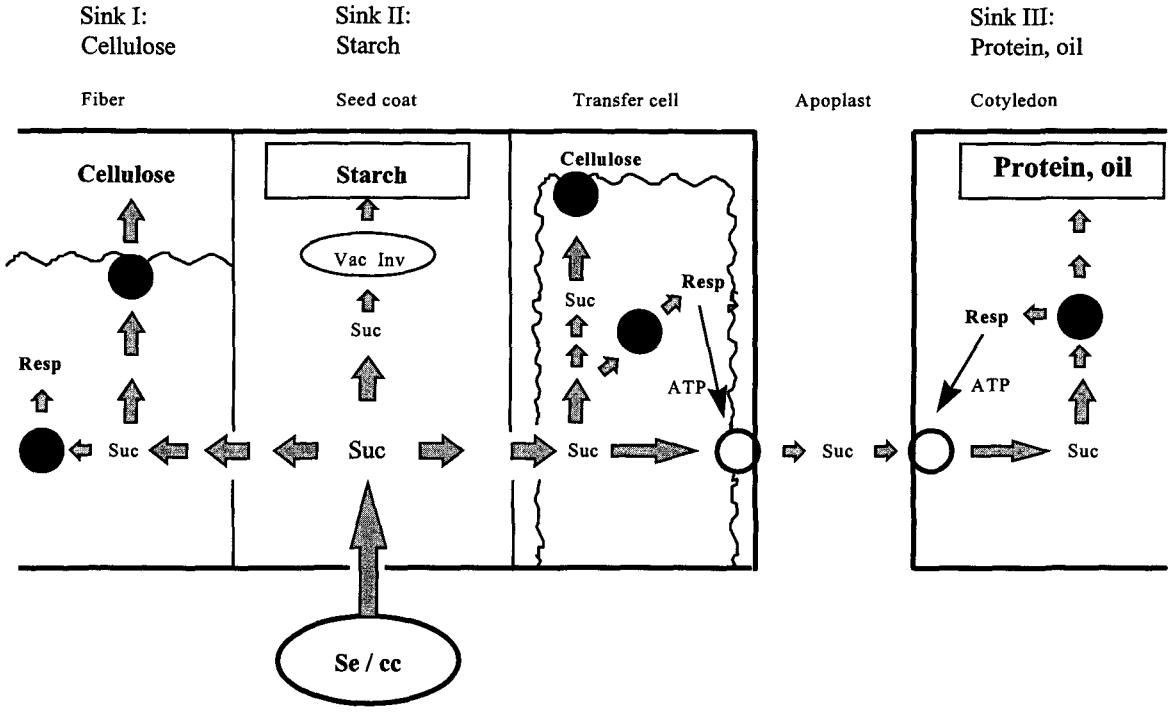

Figure 7. An integrated model for the role of SuSy in controlling diverse patterns of carbon partitioning in developing cotton seed. ●, SuSy; ○, putative Suc transporter. The arrow indicates the main direction of carbon flow. The differential expression of SuSy protein in fiber cells and transfer cells of the seed coat plays a key role in mobilizing Suc symplastically into fibers for massive cellulose biosynthesis and into transfer cells for possible energy-coupled Suc efflux into the apoplast, where it is then taken up by cotyledonary cells and degraded by SuSy for protein and oil biosynthesis. The remainder of unloaded Suc moves into seed coat cells and is degraded by vacuolar invertase for starch biosynthesis in this tissue. Inv, Invertase; Resp, respiration; Se/cc, sievel element/companion cell complex; Vac, vacuole.

suggests that photoassimilate transport to developing fiber cells follows a symplastic route. Under these conditions, only the cytoplasmic neutral or alkaline invertase and/or SuSy could play a direct role in cleavage of imported Suc (Zrenner et al., 1995). In developing cotton seeds, the cytoplasmic invertase activity is undetectable (Hendrix, 1990), which renders SuSy the principal enzyme to degrade Suc in cotton seeds (Hendrix, 1990). The fact that Suc enters cotton fiber cells without hydrolysis (Buchala, 1987) further strengthens the critical role of SuSy in degrading symplastically imported Suc for cellulose biosynthesis in developing cotton fibers.

The notion that SuSy is a key player in driving Suc to fiber cells is further sustained by the absence of SuSy in the phloem-unloading area and its presence in the fiber (Figs. 3 and 4) with a corresponding steep concentration gradient of Suc between seed coat and fiber (Table I). This gradient is probably attributable to the spatial differences in SuSy expression, particularly when Suc is mainly cytosolic (Riens et al., 1991; Heineke et al., 1994). The high activity of SuSy in fibers would lead to rapid cleavage of Suc, thus lowering the Suc concentration in these cells (Table I) to drive continuous Suc import via the symplastic pathway (Fig. 6). Such transport of Suc may be particularly critical for fibers, since the studies of Amor et al. (1995) suggest that free UDP-Glc does not serve as an efficient substrate for cellulose synthesis compared with UDP-Glc channeled directly from a membrane-associated form of SuSy.

The final striking finding is the abundance of SuSy in the innermost cell layer of the seed coat facing the cotyledons (Fig. 4, E and G). These cells contain small vacuoles, are rich in mitochondria, and produce a cell wall labyrinth resembling that of transfer cells (Ryser et al., 1988). Transfer cells in maternal tissues of developing broad beans have been found to be the principal cellular site for active Suc efflux to the seed apoplast (Patrick and Offler, 1995). The observation that SuSy protein is in great abundance in the transfer cells of the cotton seed coat supports this model in terms of energy generation by the enzyme and its role in controlling cell wall biosynthesis. Another feature of these transfer cells is that the lateral cell walls contain many plasmodesmata, providing a potential capacity for symplastic transport (Ryser et al., 1988), the functionality of which has now been demonstrated by the movement of CF (Fig. 6). Thus, unloaded Suc could also move symplastically to the transfer cells down a concentration gradient maintained by the activity of SuSy in these cells. After Suc efflux from the transfer cells into the seed apoplast, it could be taken up by the cotyledons, where SuSy is again involved in degradation of Suc for protein and lipid biosynthesis (Fig. 4; Trelease et al., 1986). Together, the abundance of SuSy in the outermost (fiber) and innermost seed coat and its deficiency in the vascular unloading region constitute a unique, priority-orientated expression pattern. This spatial expression of SuSy is likely to be the metabolic basis

for post-phloem Suc mobilization to fiber and cotyledons and thus indicates how SuSy controls sink strength. Figure 7 shows an integrated model for such a control. Overall, the developing cotton seed, with its complex patterns of carbon partitioning (Fig. 7), represents an excellent example of the diverse role SuSy can play in controlling carbon flow for multiple biosynthetic processes in a nonphotosynthetic organ.

ACKNOWLEDGMENTS

The generous supply of self-pollinated seeds of *G. hirsutum* var DH6910-24 from Dr. Richard Percy (U.S. Department of Agriculture-Agricultural Research Service, Maricopa Agriculture Center, Maricopa, AZ) is gratefully acknowledged. We thank Drs. Earl Taliercio and Susan Carlson for their critical reading of the manuscript.

Received April 9, 1997; accepted July 1, 1997.
Copyright Clearance Center: 0032-0889/97/115/0375/11.

LITERATURE CITED

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP (1995) A membrane-associated form of SuSy and its potential role in synthesis of cellulose and callose in plants. Proc Natl Acad Sci USA **92:** 9353–9357

ap Rees T (1988) Hexose phosphate metabolism by nonphotosynthetic tissues of higher plants. *In* J Preiss, ed, The Biochemistry of Plants. A Comprehensive Treatise, Vol 14: Carbohydrates. Academic Press, New York, pp 1–33

Basra A, Malik CP (1984) Development of the cotton fiber. Int Rev Cytol **89:** 65–113

Borland AM, Farrar JF (1988) Compartmentation and fluxes of carbon in leaf blades and leaf sheaths of *Poa annua* L. and *Poa jemtlandica*. Plant Cell Environ **11:** 535–543

Buchala AJ (1987) Acid β-fructofuranoside fructohydrolase (invertase) in developing cotton (*Gossypium arboreum* L.) fibers and its relationship to β-glucan synthesis from sucrose fed to the fiber apoplast. J Plant Physiol **127:** 219–230

Carlson SJ, Chourey PS (1996) Evidence of plasma membrane-associated forms of sucrose synthase in maize. Mol Gen Genet **252:** 303–310

Carpita NC, Delmer DP (1981) Concentration and metabolic turnover of UDP-glucose in developing cotton fibers. J Biol Chem **256:** 308–315

Chen YC, Chourey PS (1989) Spatial and temporal expression of the two SuSy genes in maize: immunohistological evidence. Theor Appl Genet **78:** 553–559

Cheng WH, Im KH, Chourey PS (1996) Sucrose phosphate synthase expression at the cell and tissue level is coordinated with sucrose sink-to-source transitions in maize leaf. Plant Physiol **111:** 1021–1029

Chourey PS (1981) Genetic control of sucrose synthetase in maize endosperm. Mol Gen Genet **184:** 372–376

Chourey PS, Chen YC, Miller ME (1991) Early cell degeneration in developing endosperm is unique to the *Shrunken* mutation in maize. Maydica **36:** 141–146

Chourey PS, Miller ME (1995) On the role of sucrose synthase in cellulose and callose biosynthesis in plants. *In* HG Pontis, GL Salerno, EJ Echeverria, eds, Sucrose Metabolism, Biochemistry, Physiology and Molecular Biology. American Society of Plant Physiologists, Rockville, MD, pp 80–87

Chourey PS, Nelson O (1976) The enzymatic deficiency conditioned by the shrunken 1 mutations in maize. Biochem Genet **14:** 1041–1055

Chourey PS, Nelson O (1979) Interallelic complementation at the *sh* locus in maize at the enzyme level. Genetics **91:** 317–325

Chourey PS, Taliercio EW (1994) Epistatic interaction and functional compensation between the two tissue and cell-specific sucrose synthase genes in maize. Proc Natl Acad Sci USA **91:** 7917–7921

Dellaporta SL, Wood J, Hicks JB (1983) A plant version of DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Delmer DP, Amor Y (1995) Cellulose biosynthesis. Plant Cell **7:** 987-1000

Doman DC, Walker JC, Trelease RN, Moore BD (1982) Metabolism of carbohydrate and lipid reserves in germinated cotton seeds. Planta **155:** 502–510

Geigenberger P, Stitt M (1993) Sucrose synthase catalyses a readily reversible reaction *in vivo* in developing potato tubers and other plant tissues. Planta **189:** 329–393

Goodall H, Johnson MH (1982) Use of carboxyfluorescein diacetate to study formation of permeable channels between mouse blastomeres. Nature **295:** 524–526

Heim U, Weber H, Baumlein H, Wobus U (1993) A sucrose synthase gene of *Vicia faba* L.: expression pattern in developing seeds in relation to starch synthesis and metabolic regulation. Planta **191:** 394–401

Heineke D, Wildenberger K, Sonnewald U, Willmitzer L, Heldt HW (1994) Accumulation of hexoses in leaf vacuoles: studies with transgenic tobacco plants expressing yeast-derived invertase in the cytosol, vacuole or apoplast. Planta **194:** 29–33

Heinlein M, Starlinger P (1989) Tissue- and cell-specific expression of two sucrose synthase isozymes in developing maize kernels. Mol Gen Genet **215:** 441–446

Hendrix DL (1990) Carbohydrate and carbohydrate enzymes in developing cotton ovules. Physiol Plant **78:** 85–92

King SP, Lunn JE, Furbank RT (1997) Carbohydrate content and enzyme metabolism in developing canola (*Brassica napus* L.) siliques. Plant Physiol **114:** 153–160

Kleczkowski A (1994) Glucose activation and metabolism through UDP-glucose pyrophosphorylase in plants. Plant Phytochem **37:** 1507–1515

Koßmann J, Müller-Röber B, Riesmeier J, Frommer WB, Sonnewald U, Willmitzer L (1995) Transgenic plants as a tool to analyze carbohydrate metabolism. *In* HG Pontis, GL Salerno, EJ Echeverria, eds, Sucrose Metabolism, Biochemistry, Physiology and Molecular Biology. American Society of Plant Physiologists, Rockville, Maryland, pp 100–106

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–683

Ma DP, Tan H, Si Y, Greech RG, Jenkins JN (1995) Differential expression of a lipid transfer protein gene in cotton fiber. Biochim Biophys Acta **1257:** 81–84

Mass C, Schall S, Werr W (1990) A feedback control element near the transcription start site of the maize shrunken-1 gene determines promoter activity. EMBO J **9:** 3447–3452

McElfresh K, Chourey PS (1988) Anaerobiosis induces transcription but not translation of sucrose synthase in maize. Plant Physiol **87:** 542–546

Nelson N (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem **153:** 375–380

Nolte KD, Hendrix DL, Radin JW, Koch KE (1995) Sucrose synthase localization during initiation of seed development and trichome differentiation in cotton ovules. Plant Physiol **109:** 1285–1293

Patrick JW, Offler CE (1995) Post-sieve element transport of sucrose in developing seed. Aust J Plant Physiol **22:** 681–702

Riens B, Lohaus G, Heineke D, Held HW (1991) Amino acid and sucrose content determined in the cytosolic, chloroplastic, and vacuolar compartments and in the phloem sap of spinach leaves. Plant Physiol **97:** 227–232

Ruan YL, Mate C, Patrick JW, Brady CJ (1995) Non-destructive collection of apoplast fluid from developing tomato fruit using a pressure dehydration procedure. Aust J Plant Physiol **22:** 761–769

Ruan YL, Patrick JW (1995) The cellular pathway of postphloem sugar transport in developing tomato fruit. Planta **196:** 434–444

Ryser U (1992) Ultrastructure of the epidermis of developing cotton (Gossypium) seeds: suberin, pits, plasmodesmata, and their implication for assimilate transport into cotton fibers. Am J Bot **79:** 14–22

Ryser U, Schorderet M, Jauch U, Meier H (1988) Ultrastructure of the "fringe-layer," the innermost epidermis of cotton seed coats. Protoplasma **147:** 81–90

Sonnewald U, Brauer M, von Schaewen A, Stitt M, Willmitzer L (1991) Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast: a powerful tool for studying sucrose metabolism and sink/source interactions. Plant J **1:** 95–106

Taliercio EW, Chourey PS (1989) Post-transcriptional control of sucrose synthase expression in anaerobic seedlings of maize. Plant Physiol **90:** 1359–1364

Trelease RN, Miernyk JA, Choinski JS Jr, Bortman SJ (1986) Synthesis and compartmentalization of enzymes during cotton seed maturation. *In* JR Mauney, JMD Steward, eds, Cotton Physiology. The Cotton Foundation, Memphis, TN, pp 441–462

Wadsworth GJ, Redinbaugh MG, Scandlios JG (1988) A procedure for the small-scale isolation of plant RNA suitable for RNA blot analysis. Anal Biochem **172:** 279–283

Wang F, Sanz A, Brenner ML, Smith AG (1993) Sucrose synthase, starch accumulation, and tomato fruit sink strength. Plant Physiol **101:** 321–327

Wang F, Smith AG, Brenner M (1994) Temporal and spatial expression pattern of sucrose synthase during tomato fruit development. Plant Physiol **104:** 535–540

Weber H, Buchner P, Borisjuk L, Wobus U (1996) Sucrose metabolism during cotyledon development of *Vicia faba* L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: expression patterns, metabolic regulation and implications for seed development. Plant J **9:** 841–850

Zrenner R, Salanoubat M, Willimitzer L, Sonnewald U (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). Plant J **7:** 97–107